Functional analysis of *DcpS* as a candidate cutaneous squamous cell carcinoma susceptibility gene

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ABSTRACT

DCPS is a candidate cutaneous squamous cell carcinoma (SCC) susceptibility gene as determined by allelic imbalance mapping of paired SCC and genomic blood DNA samples. DCPS shows no protein expression in 23% of human SCCs on a tissue microarray, and reduced protein expression in another 30%. This is in contrast to strong staining for DCPS in 100% of normal tissue samples. DCPS, a decapping scavenger enzyme, influences the pool of available cap-binding proteins and, in turn, impacts aspects of mRNA metabolism like pre-mRNA splicing and decay. The hypothesis driving this research is that *DCPS* acts as a tumor suppressor. To test this hypothesis, functional effects of increasing and decreasing expression of *DcpS* in a mouse keratinocyte cell line have been studied. First intron splicing and exon skipping is enhanced in *DcpS* overexpression cell lines, while splicing of the second intron is not affected by *DcpS* expression. *DcpS* knockdown cell lines were found to have more stable mRNA compared to a control cell line. *DcpS* knockdown cell lines exhibit less growth than normal and overexpression cell lines. Cell migration is not affected by *DcpS* expression. *DcpS* knockdown cell lines exhibit more apoptosis than normal and overexpression cell lines. Finally, there is a greater percentage of cells in the S and G2-M phases of the cell cycle in *DcpS* knockdown cell lines compared to mock and overexpression cell lines. From these studies it appears that while *DcpS* may affect mRNA splicing and stability, decreased levels of *DcpS* do not result in a cancer phenotype. Rather, decreased levels of *DcpS* seem to lead to an anti-tumorigenic phenotype. Future directions may include examining the effect of UV radiation on these phenotypes and determining other proteins with which DcpS interacts.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Cutaneous Squamous Cell Carcinoma (SCC)

The body's largest organ, the skin, serves as a barrier to separate internal organs from external elements such as heat, light, and numerous pathogens[1]. The skin also regulates body temperature, stores water and fat, and allows for the sense of touch. The skin is composed of three layers: epidermis, dermis, and subcutaneous fat (Figure 1). The epidermis is the thin outer layer of skin, which consists of five layers of cells in order from deep to superficial: stratum basale, stratum spinosum, stratum granulosum, statum lucidum, and stratum corneum. The germinal cells needed for regeneration of the epidermis originate in the stratum basale and undergo a progressive maturation called keratinization as they migrate to the surface.

The dermis is located between the epidermis and the layer of subcutaneous fat layer[1]. This layer contains blood vessels, lymph vessels, hair follicles, sweat glands, collagen bundles, fibroblasts, and nerves. The dermis also contains specialized cells and structures including sebaceous glands and apocrine glands, eccrine glands, Meissner's and Vater-Pacini corpuscles, and pain and touch receptors.

The deepest layer of the skin is the layer containing subcutaneous fat[1]. It consists of a network of collagen, connective tissue, and fat cells that help conserve the body's heat and protect the body from injury by acting as a shock absorber. This layer also houses larger blood vessels and nerves.

The two broad categories of skin cancer are melanoma and nonmelanoma skin cancer. Melanoma skin cancer results when melanocytes, the pigment producing cells, become malignant. Nonmelanoma skin cancer (NMSC) results from tumorigenesis of the epidermis and includes basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)[2]. This research focuses on squamous cell carcinoma. Cutaneous squamous cell carcinoma is a malignant tumor of keratinocytes in the upper layer of the epidermis[3].

Skin cancer is the world's most common cancer with more new cases annually than the combined incidences of cancers of the breast, prostate, lung, and colon[4]. There has been more than a 300 percent increase in non-melanoma skin cancer cases in the United States since 1994[4]. People who have had non-melanoma skin cancer have twice the risk of developing other malignancies. Squamous cell carcinoma is the second most common form of skin cancer, equating to 700,000 cases diagnosed annually resulting in approximately 2,500 deaths.

Cutaneous squamous cell carcinoma typically presents clinically as a new or growing lesion on the skin[3]. Tumors appear as raised, ulcerous, papules that may bleed, weep, and be painful or tender[3,5]. SCC is caused by ultraviolet radiation and most commonly occurs on the arms, legs, nose, and ears since these are the parts of the body most often exposed to the sun[6]. Other SCC risk factors include old age, smoking, human papilloma virus infection, chronic inflammation of the skin, being male, having light skin, exposure to chemical carcinogens, and immunosuppression[3,5].

1.2 Identification of *DcpS* as a candidate SCC susceptibility gene

DcpS is a candidate squamous cell carcinoma (SCC) susceptibility gene as determined by allelic imbalance mapping of SCC and genomic blood DNA samples obtained from 65 organ transplant patients[7]. Organ transplant patients tend to develop multiple tumors as a result of immunosuppression. Having multiple tumors linked to the same genomic blood DNA is important in the study of preferential allelic imbalance because it allows for determination of whether a certain allele is lost in the tumors.

Array Comparative Genomic Hybridization (aCGH) was used to determine chromosomal gains and losses in SCCs and frequency plots of these changes were mapped in over 300 SCCs[7]. Forty-five microsatellite markers were used to examine the 14 chromosomal regions showing frequent losses and gains by aCGH. Thirteen of these markers were shown to exhibit preferential allelic gains or losses in tumor/normal pairs. A few loci showing evidence of preferential allelic imbalance in tumors were targeted for higher-resolution studies to identify candidate single nucleotide polymorphisms (SNPs) driving allele-specific imbalance. SNPs mapping to a small region of about 100kb on 11q24 showed strong evidence ($p<0.005$) of preferential allelic loss. This region contains three genes: *DCPS*, *TIRAP*, and *ST3GAL4*.

DCPS was chosen to be the focus of this study because no DCPS protein expression was seen in 23% of human SCCs on a tissue microarray and another 30% showed decreased expression[7]. All normal skin samples showed strong nuclear DCPS staining. The expression pattern for ST3GAL4 was not as consistent. Human SCC samples had

similar staining patterns to normal tissue. A loss of ST3GAL4 protein expression was only detected in 2% of the samples. Protein expression of TIRAP was not examined in human SCC samples due to a lack of antibody that would bind TIRAP specifically.

1.3 DcpS Functions

DcpS, a decapping scavenger enzyme, has been shown to influence the pool of available cap-binding proteins and, in turn, impact downstream aspects of mRNA metabolism like pre-mRNA splicing, mRNA decay, nuclear export, and translation[8]. The role that DcpS has been shown to have in first intron pre-mRNA splicing provides a link between splicing and decay[9]. Human mRNA translation start sites are found in the first exon. The first intron commonly contains a premature translation termination codon, so if it is not spliced from the mRNA the nonsense-mediated mRNA decay surveillance pathway will lead to rapid degradation. When there are normal levels of DcpS in the cell, cap-binding complex (CBC) binds to the cap on the 5' end of mRNA to facilitate removal of the first intron by enhancing assembly of the splicosome[8] (Figure 3). Low DcpS levels lead to low splicing efficiency because DcpS regulates the active pool of CBC through hydrolysis of free cap structures. Free cap structures that are not attached to mRNA occupy the CBCs, inactivating them. This prevents the CBC from being available to bind to a cap structure that is attached to mRNA and promoting splicing. This was observed in rat fibronectin mini-gene mRNA as well as two multi-exon endogenous genes. As many cancers show aberrant RNA splicing, this is one potential mechanism by which DcpS might influence tumorigenesis.

Studies on *DCS1*, an orthologous gene to *DcpS* in yeast, have shown that disruption of the gene leads to a decrease in mRNA decay[8]. This effect has been rescued by exogenous expression of DCS1p, a gene with a similar function. Since the presence of DSC1p promotes decay, the cytoplasmic exoribonuclease, Xrn1p, that is responsible for the decay of decapped mRNAs seems to be the most likely target of regulation. Two theories regarding the mechanism of action have been suggested: cap structures inhibit exoribonuclease activity, or the hydrolyzed products have a stimulatory effect on exoribonuclease activity. Previously, the effect of *DCPS* on mRNA decay in mammalian cells had not been tested.

DcpS also plays a role in nuclear export through regulation of the active pool of CBC, which stimulates nuclear export[8]. Nuclear export is more efficient with higher levels DcpS. Nuclear export is important because if there is nuclear mRNA retention, there is a reduced pool of cytoplasmic mRNA available for translation. Further evidence for DcpS as a nucleocytoplasmic shuttling protein is provided by the fact that according to fluorescence studies DcpS is primarily found in the nucleus; however, it has also been isolated from the cytoplasm. DcpS contains a nuclear export signal (NES), a leucine rich region at the central hinge of the protein, and a nuclear localization signal (NLS), a stretch of basic amino acids.

Finally, DcpS affects translation by preventing the translation initiation factor eIF4E from being sequestered by free cap structures[8]. DcpS allows eIF4E to bind to 5' cap structures that are bound to mRNA and promote translation. DcpS will displace eIF4E

bound to cap structure but will not displace eIF4E from capped mRNA. This means that DcpS does not impede translation.

1.4 Hypothesis and Specific Aims

The central hypothesis driving this research is that *DcpS* acts as a tumor suppressor in cutaneous squamous cell carcinoma through its role in pre-mRNA splicing and/or mRNA stability. To test this hypothesis, functional differences that occur when *DcpS* expression is varied in a normal mouse keratinocyte cell line will be tested.

1. Determine whether differences in *DcpS* expression lead to changes in mRNA splicing and stability, which are potential mechanisms of tumorigenesis.
2. Determine whether differences in *DcpS* expression lead to functional changes in common cancer phenotypes such as cell proliferation, apoptosis, cell migration, and cell cycle stage.

A gene is considered a tumor suppressor if its loss or inactivation results in an abnormal growth of tissue[10]. When present at normal expression levels, tumor suppressor genes help regulate normal cell processes, but when they are lost or inactivated their protective effect against cancer is decreased. The decreased expression of DcpS in human SCC samples compared to normal tissue, alongside the preferential allelic imbalance data led to the hypothesis that *DcpS* acts as a tumor suppressor in cutaneous squamous cell carcinoma.

Previous research has shown that human mRNA translation start sites are found in the first exon of genes[11]. DcpS preferentially affects splicing of the first intron, and the first intron commonly contains a premature translation termination codon[8,12,13]. If the first intron is not spliced from the mRNA the nonsense-mediated mRNA decay surveillance pathway will lead to rapid degradation. Aberrant splicing has been associated with numerous cancers including lung cancer, gastric cancer, breast cancer, melanoma, and cutaneous T-cell lymphoma[14]. Altering splicing efficiency of the first intron will lead to abnormal degradation of gene products, thereby altering their expression levels. Therefore, affecting pre-mRNA splicing is a potential mechanism by which DcpS may influence tumorigenesis. DcpS may also regulate gene expression and the development of cancer through its effects on mRNA stability. Studies on *DCS1*, an orthologous gene to *DcpS* in yeast, have shown that disruption of the gene leads to a decrease in mRNA decay[15]. If mRNA remains in the cell for an abnormal amount of time, it will be available for translation for an abnormal amount of time, ultimately leading to abnormal protein levels.

In addition to determining the method by which DcpS may affect tumorigenesis, it is important to examine how changes in pre-mRNA splicing and mRNA stability relate to the function of the cells. Cancer cells frequently exhibit phenotypes such as uncontrolled proliferation, decreased apoptosis, de-regulated cell cycles, increased migration, increased invasion, and increased metastasis[10]. These phenotypes lead to the development of tumors. Since these are common cancer phenotypes, I examined

the effects of varying levels of *DcpS* expression on cell proliferation, apoptosis, cell migration, and cell cycle.

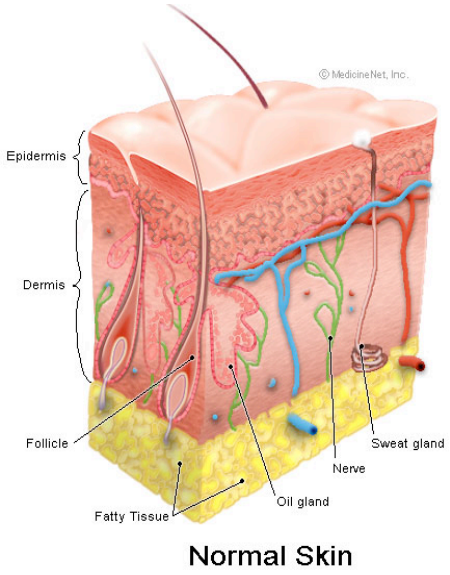


FIGURE 1. Schematic drawing of normal skin including skin layers (epidermis, dermis, and subcutaneous fat) and structures located in the skin[19].

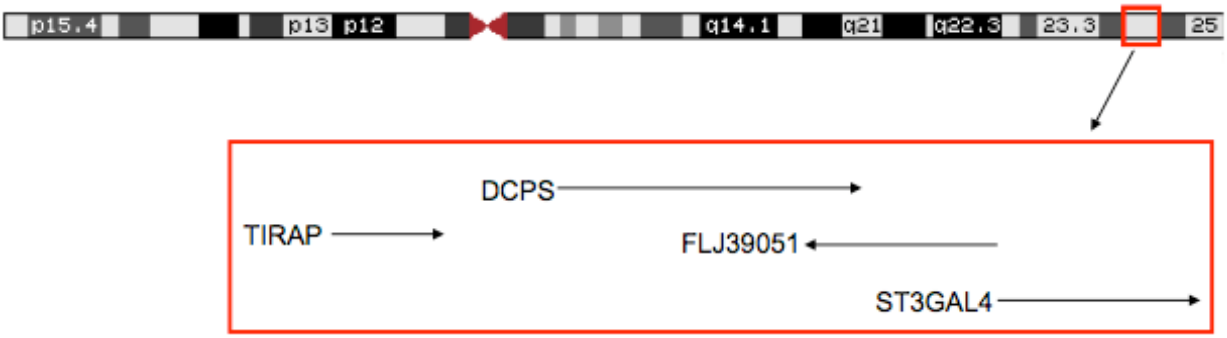


FIGURE 2. Map of 11q24, the minimal candidate region identified by Dworkin et all[7].

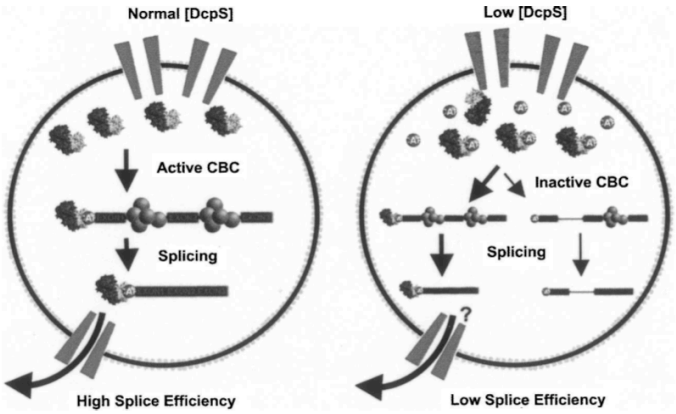


FIGURE 3. Mechanism by which DcpS influences pre-mRNA splicing efficiency. DcpS modulates the pool of active Cap Binding Complex (CBC). Active CBC binds to the 5' mRNA cap and facilitates assembly of the spliceosome at the 5' splice site of the first intron, leading to high splice efficiency. The CBC is not available to do so when it is inactivated by free cap structures[8].

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines

All experiments were performed using an immortalized normal murine keratinocyte cell line, C5N, which was obtained from Allan Balmain[16]. This cell line was directly derived from normal mouse skin. It is immortalized, but non-tumorigenic. Cells were grown in Dulbecco's modifications of Eagle's medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen) and 1% penicillin streptomycin (Invitrogen).

2.2 Transfection

Transfections were performed using Lipofectamine. The transfections for transient *DcpS* overexpression using the *DcpS* mouse cDNA clone (OriGene), pCMV6-Kan/Neo, were performed according to the Lipofectamine protocol in a final volume of 5 mL of OPTI-MEM in a 10 cm dish (Invitrogen). C5N cells were plated prior to transfection in a 10 cm dish. Cells were transfected at 70% confluency.

2.3 Lentiviral Transduction

Lentiviral transduction was used to make stable *DcpS* knockdown cell lines. The 293RTV cell line was used to grow the virus (Cell Biolabs). This cell line is a permanent line established from primary embryonic human kidney and transformed with human adenovirus type 5 DNA. Four different mouse pLKO.1 shRNA constructs against *DcpS* were used separately and in combination to achieve knockdown (Open Biosystems). The packaging plasmid, pCMV-dR8.2, and the envelope plasmid, pMD2.D, were also

transfected (addgene). Transfections with *DcpS* shRNAs were performed according to the FuGENE 6 protocol (Roche) in a final volume of 4 mL of Dulbecco's modifications of Eagle's medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen) and 1% penicillin streptomycin (Invitrogen) in a 6cm dish. 293RTV cells were plated prior to transfection in the 6cm dish. Cells were transfected at 70% confluency. After incubating cells for 18 hours (37C, 5% CO_2) media was changed to 6 mL high serum growth media (30% FBS). Viral supernatant was collected over the next 30 hours. C5N cells plated in 6 mL complete media in 6cm dishes were treated with viral supernatant at 70% confluency. Puromycin (Enzo Life Sciences) selection was begun 32 hours post-infection at a concentration of 1.5ug/mL. 12 days post-selection antibiotic, Puromycin concentration was reduced to 0.75ug/mL.

2.4 Pre-mRNA Splicing

DcpS overexpression, knockdown, and mock cell lines were transfected with a mini-gene construct, pCMV2B MDM2 3-11-12[20], according to Lipofectamine protocol (Invitrogen). Whole-cell RNA was extracted from cells and reverse transcribed into cDNA. Primers specific to the mini-gene were used to amplify cDNA, which was then analyzed using gel electrophoresis. The amount of each splicing product was quantitated using Alpha Imager.

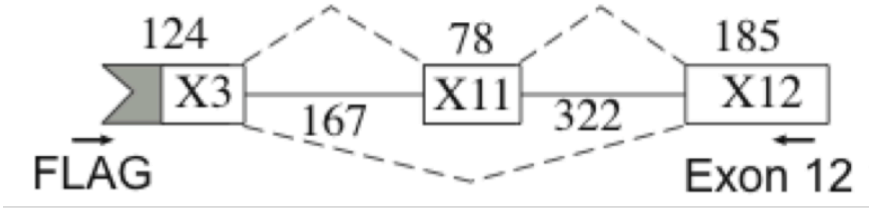


FIGURE 4. Diagram of pCMV2B MDM2 3-11-12 mini-gene construct, including number of base pairs in each intron and exon[20].

2.5 mRNA Stability

A *DcpS* knockdown cell line and overexpression cells, forty-eight hours after transfection, were treated with actinomycin D and alpha-amanitin, to prevent RNA production, and were harvested at 0, 2, 4, 8, and 24 hours. Whole-cell RNA was extracted from cells and reverse transcribed into cDNA. RT-qPCR was used to measure mRNA levels of 4 genes, *Ahr* (2.51h half-life), *Twistnb* (3.03h half-life), *Hprt1* (8.46h half-life), and *Ppia* (9.19h half-life)[21]. mRNA levels at each time point were calculated as a percentage of mRNA at t=0 treated.

2.6 Cell Proliferation

One day post-transfection, *DcpS* overexpression cells and a *DcpS* knockdown cell line in 10 cm dishes were scraped, and cells were replated in quadruplicate into a 96 well dish. Proliferation was measured at 24, 48, and 72 hours post-transfection using the MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Proliferation Kit I according to manufacturer's instructions (Roche). The cells were solubilized four hours after the addition of the MTT reagent using the kit's provided solubilization reagent. The

solution was then incubated for 24 hours, and absorbance was then measured at 550 nm using a spectrophotometer.

2.7 Apoptosis

Apoptosis of experimental cell lines was determined using flow cytometry. Cells were treated with 0.05% EDTA (Ethylenediaminetetraacetic acid) and 0.1% trypsin, separated into single cells using filters, washed with PBS, and resuspended in 1x Binding Buffer at a concentration of 2×10^6 cells/mL. Cells were stained with FITC Annexin-V and Propidium iodide. Annexin-V is used to detect the presence of a marker of early apoptosis. Propidium iodide is used to visualize DNA in the nucleus, and is therefore a marker of late apoptosis. Samples were analyzed by fluorescence activated cell sorting (FACS).

2.8 Cell Migration

DcpS overexpression, knockdown, and mock cell lines were plated in migration wells, which create a cell-free gap, as in scratch assays, of approximately 500 um at a concentration of 7×10^5 cells/mL (ibidi). 70 uL of this solution was placed in each well and incubated overnight at 37C and 5% CO_2. Migration wells were then removed and cells were able to fill the cell-free gap. Migration was monitored at approximately 0, 12, and 24 hours.

2.9 Cell Cycle Stage

Cell cycle stage of experimental cell lines was determined using flow cytometry 48 after transfection. Cells were treated with 0.05% EDTA and 0.1% trypsin, separated into single cells using filters, washed with PBS, and resuspended in cold 70% ethanol. RNAase A was added to remove any residual RNA. The DNA was stained with propidium iodide, in order to visualize DNA content, and therefore the cell cycle stage. Samples were analyzed by FACS.

2.10 Quantitative Real-Time PCR

Whole-cell RNA was extracted from experiments using RiboZol Extraction Reagent (AMRESO). cDNA was generated from extracted RNA using the I-Script kit according to manufacturer's protocol (BioRad). Transfection efficiency was verified by measuring *DcpS* expression at experimental timepoints post-transfection using quantitative real-time PCR (qPCR).

Taqman qPCR was performed in triplicate according to manufacturer's protocol to measure *DcpS* expression (Applied Biosystems). *DcpS* expression was presented as a percent relative expression to *Gapdh* expression using the ΔCT equation (equation: (2^X)*100 where x= control gene expression-target gene expression). Probes were obtained via Applied Biosystems.

2.11 Western Blotting

Protein was extracted at experimental timepoints via solubilization of the cells in RIPA buffer (50 mM Tris base pH 8, 150 mM NaCl, 1% NP40, 0.10% SDS) and subsequent removal of the supernatant (20 uL). Equal amounts of protein (30 ug each) were separated by 10% SDS-PAGE and then transferred to nitro-cellulose membrane. The membranes were blocked with a buffer containing 5% nonfat milk in phosphate-buffered saline with 0.05% Tween 20 for 30 min and incubated overnight with a 1:200 dilution of DcpS primary antibody (Santa Cruz Biotechnology). After 3 washes with phosphate-buffered saline with 0.05% Tween 20, the membranes were incubated with an anti-mouse secondary antibody for 2 hours. α-tubulin (Santa Cruz Biotechnology) was used as a loading control.

2.12 Calculations and Statistical Significance

Averages and standard deviations were determined for experimental and control variables using Microsoft Excel. A two-tailed student's t-test was used to determine significance. Differences between variables were considered significant if $p<0.05$.

2.13 Primers: Table 1

Amplified DNA	Forward	Reverse
MDM2 mini-gene intron 1	5’ GAT TAC AAG GAT GAC GAT AAG 3’	5’ CCC TGC CTG ATA CAC AGT A 3’
MDM2 mini-gene intron 2	5’ GTT ACT GTG TAT CAG GCA GG 3’	5’ CAA TCA GGA ACA TCA AAG CC 3’
p53 mini-gene intron 1	5’ GAT TAC AAG GAT GAC GAT AAG 3’	5’ CGG AGA TTC TCT TCC TCT GT 3’
p53 mini-gene intron 2	5’ AGC TTT GAG GTG CGT GTT TGT GCC T 3’	5’ CCC TCG AGC TGA AGG GTG AAA TAT TCT CCA TCC 3’

TABLE 1. Primer sets used for PCR amplification in pre-mRNA splicing experiments[20].

CHAPTER 3

RESULTS

3.1 *DcpS* enhances pre-mRNA splicing

To determine if *DcpS* affects pre-mRNA splicing in mammalian keratinocytes, an MDM2 mini-gene was transfected into *DcpS* overexpression, *DcpS* knockdown, and mock C5N cell lines. Splicing products were quantified using Alpha Imager (Figure 5). The first intron was retained in 13.84% of splicing product from the mock cell line and 7.07% of splicing product from the *DcpS* overexpression cell line (Figure 5A). A similar trend of decreased intron retention in DcpS overexpression cell lines was observed in 4/4 experimental replicates. The second intron was retained in 39.43% of mock cell line splicing product and 37.89% of DcpS overexpression splicing product (Figure 5B). 3/3 experimental replicates showed similar findings. 38.81% exon skipping was observed in *DcpS* overexpression cell lines compared to 20.95% exon skipping in the mock cell line (Figure 5C). This trend was observed in 3/3 repeats of the experiment. Splicing results of the first intron, second intron, and exon skipping for *DcpS* knockdown cell lines compared to mock were highly variable. Overall, there is approximately 61.63% removal of both introns in mock cell lines, compared to 65.30% removal of both introns in the *DcpS* knockdown cell line (Figure 5D). These results suggest that increased *DcpS* enhances splicing of the first intron and exon skipping, while splicing of the second intron is not affected.

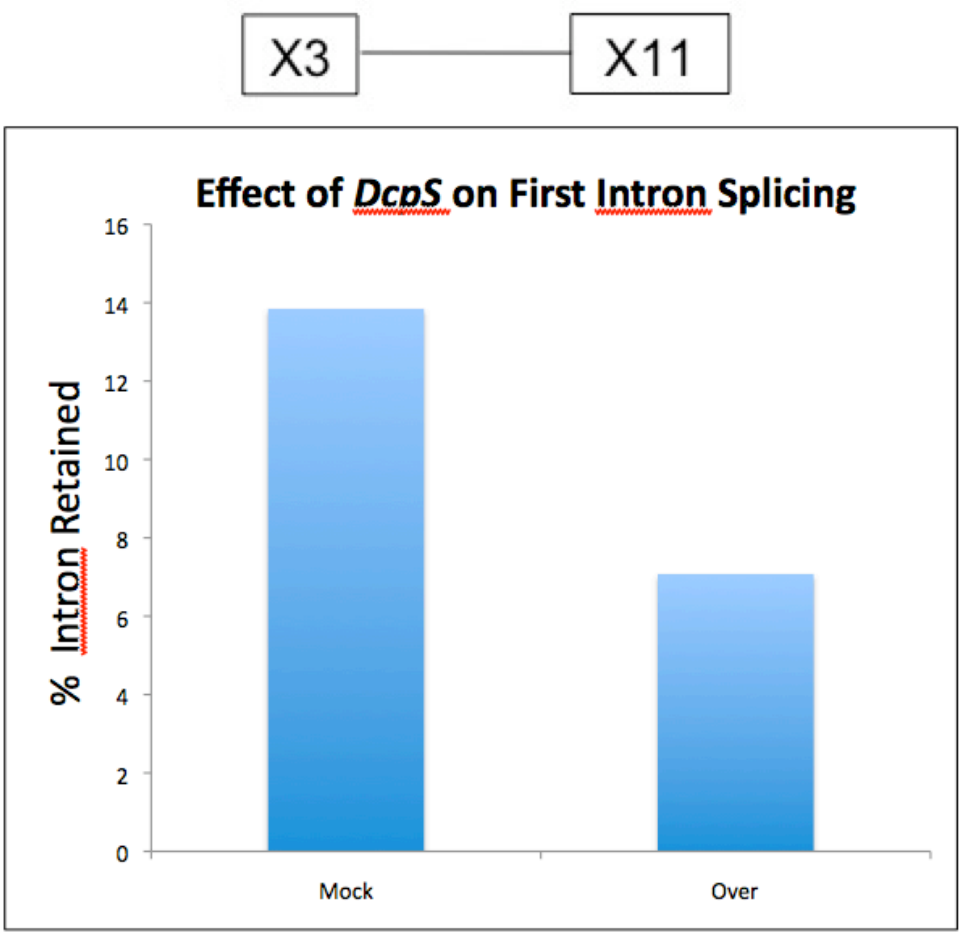


FIGURE 5A. Above: Diagram of the splicing product that is quantified in this figure. Below: Splicing of the first intron was measured in *DcpS* overexpression (over) and mock cell lines by quantifying cDNA amplified using primers specific for the first intron of the mini-gene.

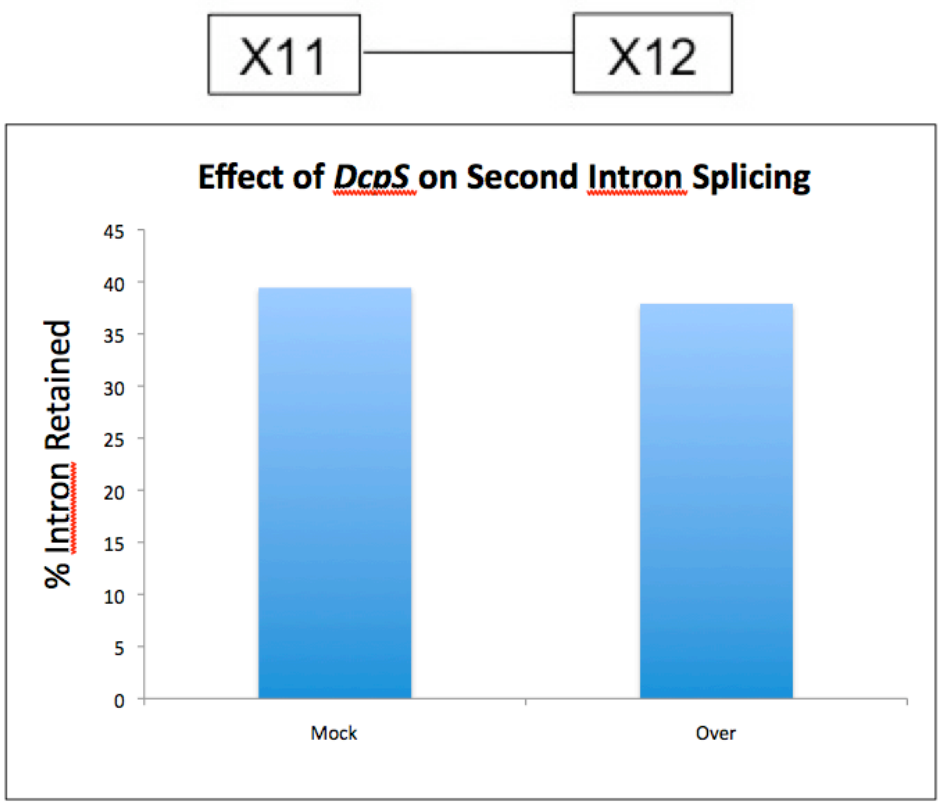


FIGURE 5B. Above: Diagram of the splicing product that is quantified in this figure. Below: Splicing of the second intron was measured in *DcpS* overexpression (over) and mock cell lines by quantifying cDNA amplified using primers specific for the second intron of the mini-gene.

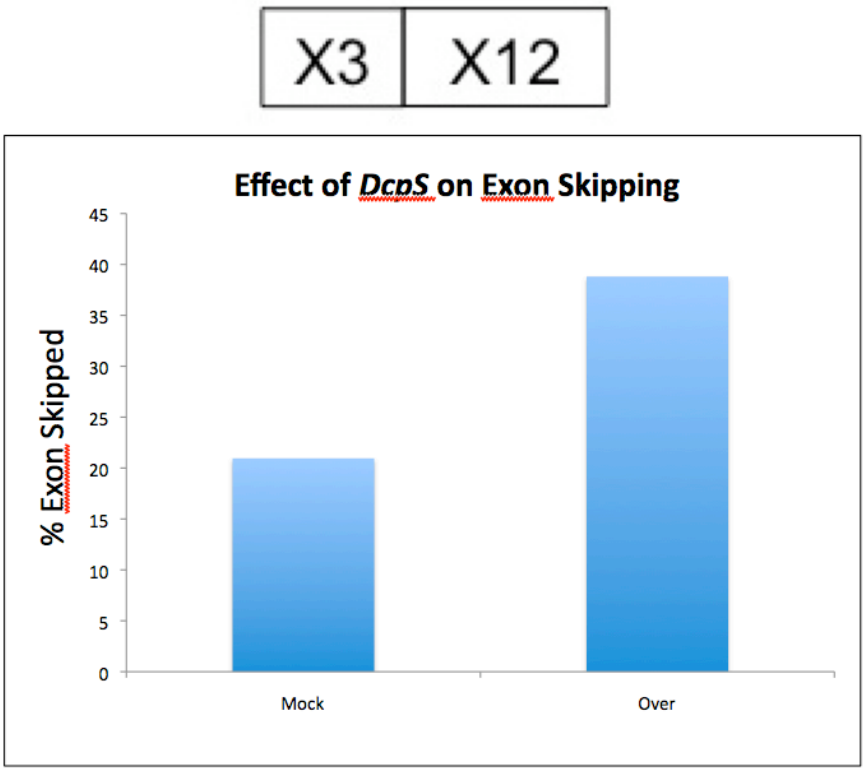
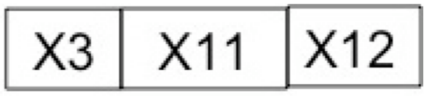


FIGURE 5C. Above: Diagram of the splicing product that is quantified in this figure.

Below: Exon skipping was measured in *DcpS* overexpression (over) and mock cell lines by quantifying cDNA amplified using primers that spanned the entire mini-gene.



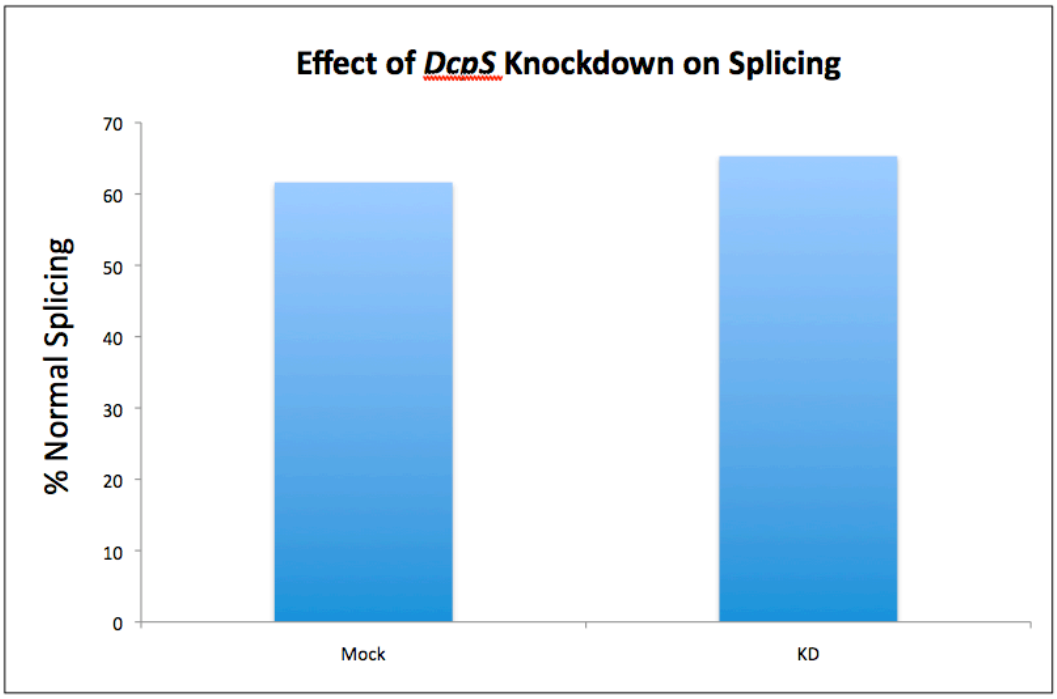


FIGURE 5D. Above: Diagram of the splicing product that is quantified in this figure. Below: The effect of *DcpS* knockdown (KD) on splicing was measured by quantifying cDNA amplified using primers spanning the entire mini-gene.

3.2 Loss of *DcpS* increases stability of mRNA

To determine if the amount of DcpS in a cell influences the stability of mRNA, *DcpS* overexpression, *DcpS* knockdown, and mock C5N cell lines were treated with Actinomycin D and alpha-amanitin to stop the production of mRNA. RNA from genes with short and medium length half-lives was then measured at 0, 2, 4, 8, and 24 hours after treatment using RT-qPCR (Figure 6). *Ahr* (2.51h half-life)[21] had significantly more mRNA 4 and 8 hours after treatment in a *DcpS* knockdown cell line compared to mRNA levels in mock and DcpS overexpression cell lines ($p < 0.05$; Figure 6). *Hprt1* (8.46h half-life)[21] had significantly more mRNA 2, 4, 8, and 24 hours after treatment in a *DcpS* knockdown cell line compared to mRNA levels in mock and DcpS overexpression cell lines ($p < 0.05$; Figure 6). Significance at the 24-hour time point was not repeated in 4/6 experiments. *Ppia* (9.19h half-life)[21] mRNA showed similar trends to those illustrated with *Ahr* and *Hprt1*. These results suggest that *DcpS* knockdown increases mRNA stability, particularly at early time points of the experiment.

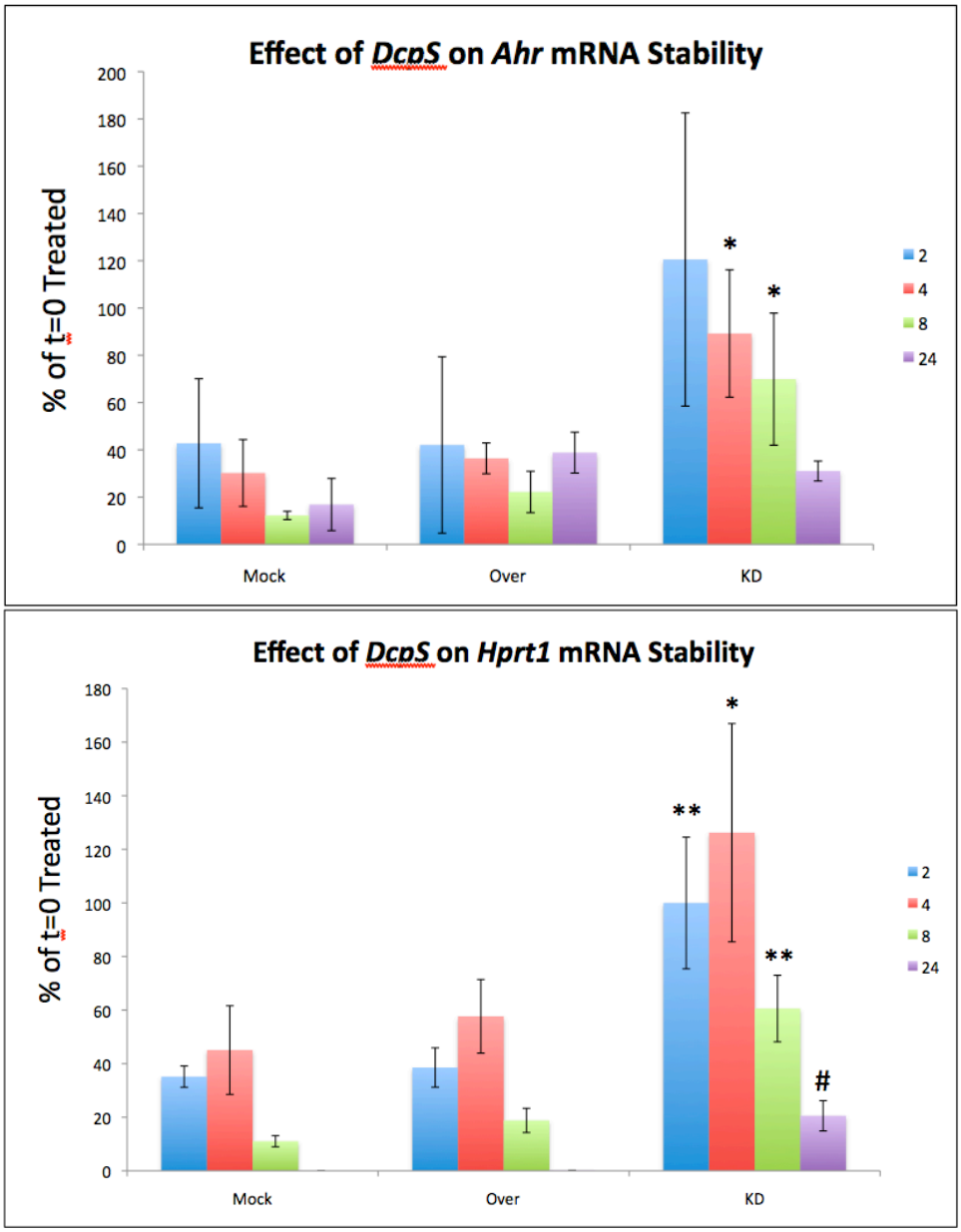


FIGURE 6. *Ahr* and *Hprt1* mRNA stability was measured via RT-qPCR at 0, 2, 4, 8, and 24 hours in *DcpS* overexpression (over), *DcpS* knockdown (KD), and mock C5N cell lines. mRNA stability was measured in triplicate. *$p < 0.05$. **$p < 0.01$. #$p < 0.01$, significance was not detected in experimental replicates.

3.3 Loss of *DcpS* reduces cell proliferation

To determine if DcpS affects cell proliferation, *DcpS* overexpression, *DcpS* knockdown, and mock C5N cell lines were used to measure cell proliferation at 24, 48, and 72 hours post transfection via an MTT assay (Figure 7). A significant decrease in growth rate (slope) was observed from 24 to 72 hours in *DcpS* knockdown cells compared to the mock and *DcpS* overexpression cell lines ($p < 0.01$; Figure 7). Specifically, a significant decrease in growth rate (slope) was observed from 24 to 48 hours in *DcpS* knockdown cells compared to the mock and *DcpS* overexpression cell lines ($p < 0.01$; Figure 7). The difference in growth rate (slope) from 48 to 72 hours between *DcpS* knockdown cells compared to the mock was not significant ($p=0.062$). Similar results were observed in two out of three total experiments. These results suggest *DcpS* knockdown reduces cell proliferation.

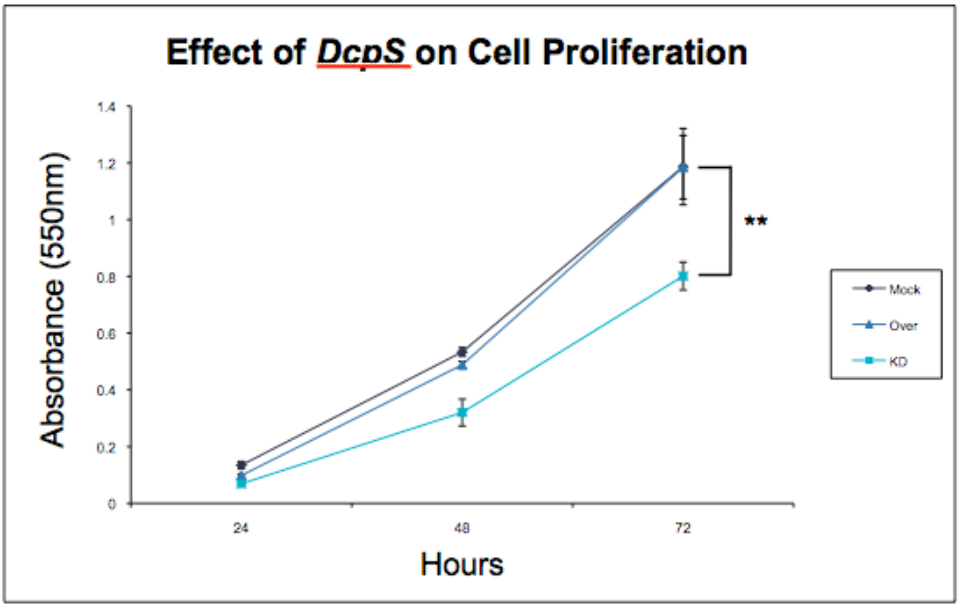


FIGURE 7. Cell proliferation (Absorbance 550nm) was measured via an MTT assay at 24, 48, and 72 hours in *DcpS* overexpression (triangle), *DcpS* knockdown (square), and mock (diamond) C5N cell lines. Proliferation at each time point was measured in quadruplicate. **p < 0.01.

3.4 *DcpS* does not affect cell migration

To determine whether *DcpS* affects cell migration, *DcpS* overexpression, *DcpS* knockdown, and mock C5N cell lines were plated in migration wells, which created a cell-free gap as in a scratch assay (Figure 8). Pictures were taken of cells approximately every 12 hours to monitor migration of cells towards closing the gap. Using this method, no consistent difference in cell migration between cell lines could be detected. In two out of four total experiments no difference was detected. In one out of four experiments, *DcpS* knockdown cells demonstrated faster migration. In one out of four experiments, *DcpS* overexpression cells showed faster migration.

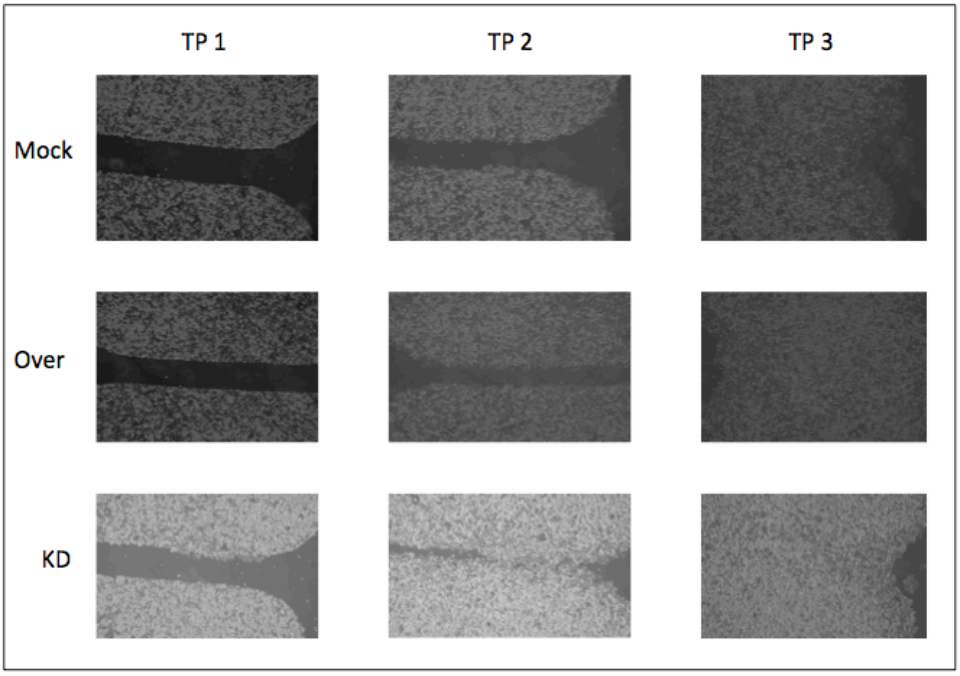


FIGURE 8. Cell migration was monitored at approximately 0, 12, and 24 hours via scratch assay in *DcpS* overexpression (over), *DcpS* knockdown (KD), and mock C5N cell lines. Shown here are representative pictures of the migration observed.

3.5 Loss of *DcpS* induces apoptosis

To determine if *DcpS* affects apoptosis, *DcpS* overexpression, *DcpS* knockdown, and mock C5N cell lines were stained with FITC Annexin-V and propidium iodide then analyzed by FACS (Figure 9). A significant increase in apoptosis was observed in *DcpS* knockdown cells compared *DcpS* overexpression and mock cell lines ($p < 0.05$; Figure 9). These results suggest DcpS induces apoptosis, which, in part, may account for the observed decrease in cell proliferation. Four *DcpS* knockdown cell lines generated using shRNAs targeted to different regions of *DcpS* were tested. Three of the four stable *DcpS* knockdown cell lines consistently showed a trend of increased apoptosis.

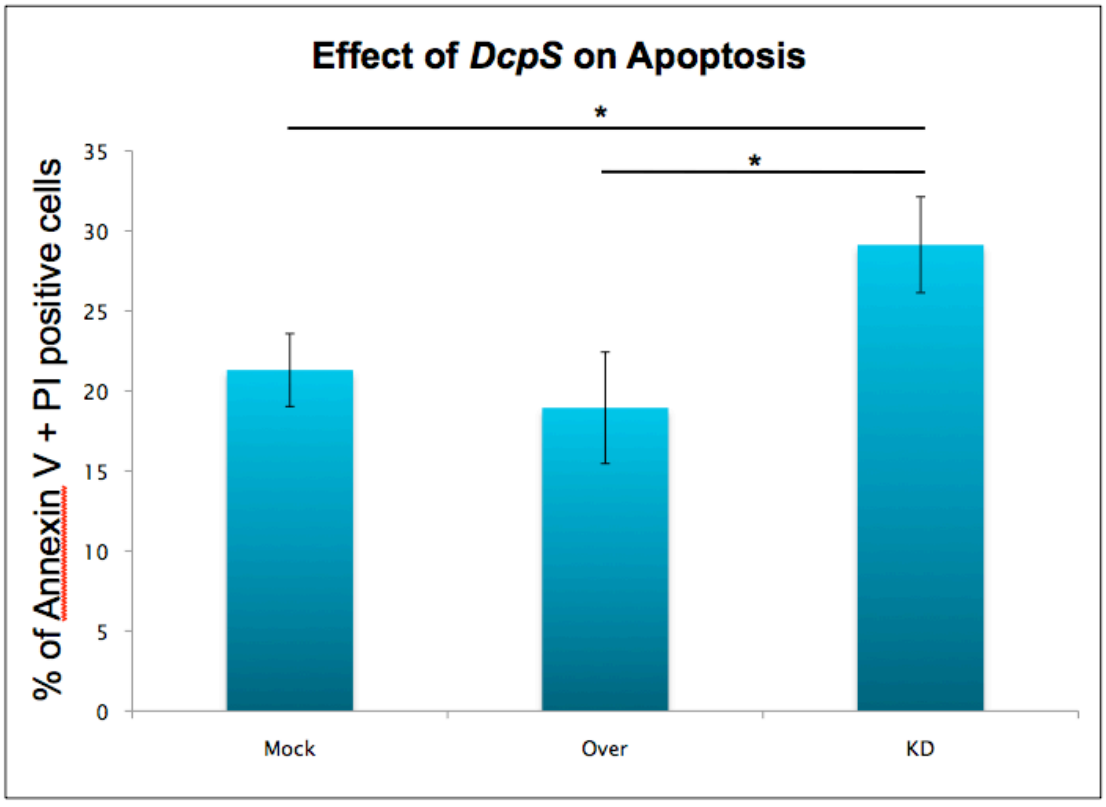


FIGURE 9. Apoptosis (stained with FITC Annexin-V and Propidium Iodide) was measured via flow cytometry using mock, *DcpS* overexpression (over), and *DcpS* knockdown (KD) C5N cell lines. Apoptosis was measured in triplicate. *$p < 0.05$.

3.6 Loss of *DcpS* increases the percentage of cells in S phase and G2-M phase

To determine if *DcpS* affects cell cycle stage, *DcpS* overexpression, *DcpS* knockdown, and mock C5N cell lines were stained with propidium iodide to visualize DNA content and analyzed by FACS 48 hours after transfection (Figure 10). A significant increase in the percentage of cells in S phase was observed in *DcpS* knockdown cells compared *DcpS* overexpression and mock cell lines ($p < 0.01$; Figure 10). There was also a significant increase in the percentage of cells in G2-M phase of the cell cycle in *DcpS* knockdown cells compared *DcpS* overexpression and mock cell lines ($p < 0.01$; Figure 10). Out of three experimental replicates using four *DcpS* knockdown lines generated using different shRNAs, increased S and G2-M phase was seen in 6/12, no change was observed in 4/12, and 2/12 showed a decreased percentage of cells in the S and G2-M cell cycle stage. Figure 10 shows a significant decrease in the percentage of cells in both S phase and G2-M phase in *DcpS* overexpression cells compared to a mock cell line ($p < 0.05$; Figure 10). This result was not detected in 2/3 experimental replicates.

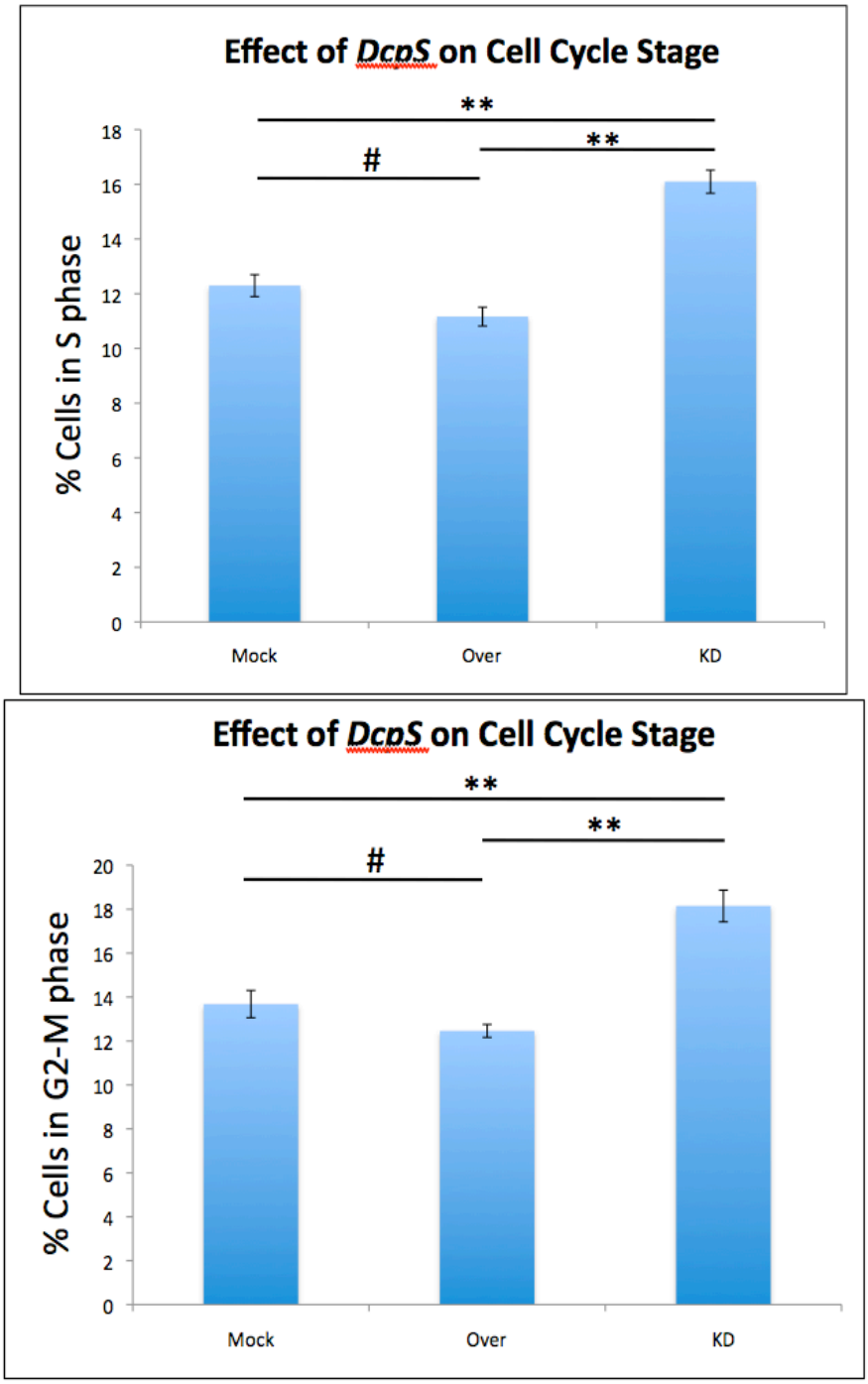


FIGURE 10. Cell cycle stage (stained with Propidium Iodide) was monitored via flow cytometry using mock, *DcpS* overexpression (over), and *DcpS* knockdown (KD) C5N cell lines. Cell cycle stage was measured in triplicate. **$p < 0.01$. #$p < 0.05$, significance was not detected in experimental replicates.

CHAPTER 4

DISCUSSION

We hypothesized that *DcpS* acts as a tumor suppressor in cutaneous squamous cell carcinoma through its effects on pre-mRNA splicing and mRNA stability. Furthermore, we tested the effects of increasing and decreasing the expression of *DcpS* on common cancer phenotypes such as cell proliferation, apoptosis, cell migration, and cell cycle stage to determine functional differences of altered *DcpS* expression. In summary, there were two aims to this study:

1. Determine whether differences in *DcpS* expression lead to changes in mRNA splicing and stability, which are potential mechanisms of tumorigenesis.
2. Determine whether differences in *DcpS* expression lead to functional changes in common cancer phenotypes such as cell proliferation, apoptosis, cell migration, and cell cycle stage.

4.1 *DcpS* alters pre-mRNA splicing and mRNA stability

Based on our results, *DcpS* overexpression transfection results in enhanced splicing of the first intron of the MDM2 mini-gene as well as increased exon skipping of the MDM2 mini-gene. *DcpS* knockdown cell lines did not alter splicing of the mini-gene compared to a mock cell line. In addition, we have shown increased mRNA stability with *DcpS* knockdown of the genes *Ahr*, *Ppia*, and *Hprt1*, particularly at early time points. *DcpS* overexpression was not determined to have an effect on mRNA stability. These results together suggest *DcpS* may be regulating protein levels in the cell through two potential mechanisms.

Previous studies have shown that *DcpS* knockdown decreased splicing efficiency of the first intron of a rat fibronectin mini-gene mRNA as well as two multi-exon endogenous genes[8]. While we did not see these effects with *DcpS* knockdown, we did show that increasing DcpS affects splicing of the first intron. We may not have seen effects with *DcpS* knockdown because the published study achieved greater than 90% knockdown, while the knockdown cell lines used in this study have 50-60% knockdown of *DcpS*. There may also be a cell-line specific component to the splicing effect that contributed to the difference in results, since human embryonic kidney cells were used previously and this study is examining mouse keratinocytes. A further limitation of this study is the fact that it is not known whether there is a particular family of genes that is affected by DcpS, while others are not as affected. If genes that are unaffected by DcpS are chosen for study, the true effect of DcpS on pre-mRNA splicing within the cell will not be realized. Pre-mRNA splicing may influence protein levels because human mRNA translation start sites are commonly found in the first exon[9]. The first intron commonly contains a premature translation termination codon, so if it is not spliced from the mRNA the nonsense-mediated mRNA decay surveillance pathway will lead to rapid degradation. Based on the enhancement of splicing observed with *DcpS* overexpression, it seems clear that *DcpS* influences pre-mRNA splicing.

The mRNA stability results obtained in this study are consistent with a previous study conducted on an orthologus gene, *DCS1*, in yeast[15]. This study showed that disruption of the gene leads to an increase in mRNA stability, just as this study demonstrated that

DcpS knockdown lead to more stable mRNA. This indicates that this gene plays a role in mRNA stability in both non-mammalian and mammalian systems. It had not previously been established that DcpS may have a greater effect on mRNA stability at earlier time points. This suggests that there are other proteins involved in the stability of mRNA at later time points. This study was limited by not having an internal control gene for RT-qPCR to which the test genes could be normalized. Since all mRNA production is stopped in the cell by Actinomycin D and alpha-amanitin, all mRNA will experience some degradation. An alternative to using mRNA to normalize to is to use ribosomal RNA (rRNA) such as 18S. This approach was attempted however; Actinomycin D inhibits the production of rRNA. mRNA production was not shown to be inhibited using alpha-amanitin alone, so it was necessary to use it in conjunction with Actinomycin D. Changes in mRNA stability may influence protein levels because mRNA transcripts are present in the cell for abnormal amounts of time. This means they are available for translation for an abnormal amount of time, leading to disregulated protein levels.

In regard to the effects of *DcpS* on both pre-mRNA splicing and mRNA stability, it will be important to determine whether *DcpS* has a global affect, or if there is a specific subset of genes that *DcpS* affects preferentially. For pre-mRNA splicing this may be accomplished by using a microarray in which there is a “common” probe that determines whether a gene is expressed, and an “isoform-specific” splice junction and exon probe set to distinguish alternative mRNA isoforms[17]. This would be used to measure differences in the abundance of different isoforms in *DcpS* overexpression, *DcpS* knockdown, and mock C5N cell lines and to determine which genes *DcpS* affects.

Alternatively, RNA-seq libraries generated from *DcpS* overexpression, *DcpS* knockdown, and mock C5N cell lines may be used to determine whether there are splicing differences between cell lines with different levels of *DcpS*. Analysis of this data will yield information about how the splicing of all transcribed genes varies as levels of *DcpS* change.

Similar results may be obtained for mRNA stability by incubating experimental cell lines in growth media containing 4-thiouridine, which is incorporated into cellular RNA[18]. 4-thiouridine-labelled transcripts would then be removed from 4-thiouridine containing growth media and quantified at different time points. A relative decay rate can be calculated for each transcript based on the rate at which the 4-thiouridine labeled transcripts, in the absence of 4-thiouridine media, are replaced by newly synthesized unlabelled mRNAs.

4.2 *DcpS* Affects Common Cancer Phenotypes

Based on our results, *DcpS* knockdown using an shRNA results in a decrease in growth rate between 24 and 48 hours in *DcpS* knockdown cells compared to mock and *DcpS* overexpression cell lines. In addition, apoptosis was significantly increased in *DcpS* knockdown cells compared to mock and *DcpS* overexpression cell lines. This suggests that a decrease in DcpS, at least in part, may be decreasing cell proliferation by inducing apoptosis. Cell cycle analysis showed that *DcpS* knockdown lead to a significant increase in the percentage of cells in S phase and G2-M phase, meaning that more cells are synthesizing DNA and dividing. Combined with the cell proliferation and

apoptosis findings, this suggests that with a loss of *DcpS* more cells are trying to grow, but they are undergoing apoptosis, which ultimately leads to a decrease in cell proliferation. This is opposite what we would have expected to find if *DcpS* were acting as a tumor suppressor as originally hypothesized. If this were the case, we would have expected *DcpS* knockdown to ultimately increase cell proliferation, likely by decreasing apoptosis and increasing the percentage of dividing cells. Knockdown of DcpS may have the observed phenotype because it is not the gene at 11q24 driving susceptibility to SCC, because DNA damage is required to see tumorigenic effects, because DcpS works with another protein that must be lost or mutated to lead to the development of cancer, or DcpS is involved with tumor progression rather than initiation.

While consistent differences in cell migration due to *DcpS* expression were not detected in this study, a more sensitive screen has the potential to reveal such effects. Changing the observation interval from 12 hours to something shorter such as 3 hours or continuous monitoring would be the best way to detect changes in cell migration due to *DcpS* levels, since there does not seem to be any gross abnormalities. Alternatively, cell migration may be studied by plating experimental cell lines on fibronectin-coated membranes and allowing them to migrate for 6 or 12 hours. Following migration, cells would be fixed, crystal violet stained, and cells on the top of the membrane would be removed. Sorenson's buffer would be added to the migrated cells to release crystal violet staining and absorbance of the stain would be measured by luminometer. Cell migration levels of each of the experimental cell lines would be compared based on absorbance.

4.3 Future Studies and Conclusion

Based on the results of this study and the fact that there are other genes in the 11q24 region that are also candidate susceptibility genes, it is unlikely that *DcpS* is a susceptibility gene for cutaneous squamous cell carcinoma. However, the possibility remains that *DcpS* is acting in conjunction with other factors to lead to the development of SCC.

One of these factors contributing to the development of SCC may be exposure to UV radiation or other carcinogens. A future study to address this possibility would be to expose samples to UV radiation prior to conducting experiments on the common cancer phenotypes discussed here: cell proliferation, apoptosis, cell migration, and cell cycle stage. There may be new or more significant differences between *DcpS* overexpression, *DcpS* knockdown, and mock C5N cell lines in this circumstance.

Another possibility is that DcpS interacts with one or more additional proteins to lead to the developments of SCC. In this case, the interacting proteins may need to be lost or mutated in addition to a loss of DcpS in order to observe the cancer phenotype. Proteins that directly interact with DcpS may be determined through a co-immunoprecipitation experiment in which antibody would be used against DcpS with the hope of isolating a larger interacting complex. In order to examine a panel of genes that may be affected by *DcpS* expression, but not necessarily directly interacting with DcpS,

would require the use of a microarray experiment comparing differences between gene expression levels in *DcpS* overexpression, *DcpS* knockdown, and mock C5N cell lines.

Finally, *DcpS* may be involved with the progression of tumors, rather than in their initiation. In order to further examine this possibility, it would be necessary to use an *in vivo* model of SCC. Using this model, *DcpS* expression could be monitored throughout the progression and development of a tumor. In addition, after the initiation of tumors, *DcpS* could be added or removed and the effect could be observed. Mice who are heterozygous for *DcpS* or mice that have *DcpS* knocked out selectively in keratinocytes could also be used to study the role of *DcpS* in SCC.

In conclusion, I have found that *DcpS* seems to affect pre-mRNA splicing and mRNA stability. These findings confirm results that had been seen previously by other studies[9,15]. However, while *DcpS* does affect pre-mRNA splicing and mRNA stability, it does not appear to be acting as a tumor suppressor in SCC. In fact, loss of *DcpS* leads to anti-tumorigenic phenotypes such as deceased cell proliferation and induced apoptosis. Based on the results from this study, it seems unlikely that *DcpS* is important in cutaneous squamous cell carcinoma susceptibility, however further studies are needed to definitively determine whether these characteristics of *DcpS* will continue to be detected in *in vivo* studies. If loss of *DcpS* does indeed have anti-tumorigenic properties, this will be important information for the development of future cancer susceptibility screens and therapeutic treatments.

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